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AU Gillardon F; Moll I; ***Uhlmann E***
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TI Antisense oligodeoxynucleotides to bax mRNA promote survival of rat sympathetic neurons in culture.
AU Gillardon F; Zimmermann M; ***Uhlmann E*** ; Krajewski S; Reed J C; Klimaschewski L
SO JOURNAL OF NEUROSCIENCE RESEARCH, (1996 Mar 15) 43 (6) 726-34.

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Binding of Antisense Phosphorothioate Oligonucleotides to Murine Lymphocytes Is Lineage Specific and Inducible

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ABSTRACT

A phosphorothioate oligonucleotide that has been employed to inhibit HIV-1 viral expression in chronically infected H9 cells was examined for its ability to associate with murine lymphoid cells. The relationship between cellular oligonucleotide concentration and the lymphoid target tissues is important to the selection of an animal model, evaluation of potential side effects, and understanding the actions of a therapeutically useful antisense oligonucleotide. Lymphoid cells were harvested from murine peripheral blood, bone marrow, thymus, lymph node, and spleen. Cell subpopulations that bind the oligonucleotide were distinguished by two-color flow cytometry employing a fluorescein-labeled anti-*rev* oligonucleotide and phycoerythrin-labeled antibodies to selected cell surface molecules associated with unique subpopulations of cells. Very little oligonucleotide binding was observed in peripheral blood mononuclear cells or thymic T cells, but substantial numbers of cells, primarily B cells from bone marrow and spleen, accumulated the oligonucleotide. The cell-associated oligonucleotide was increased significantly in lymphoid populations when the cells were mitogen pretreated with either concanavalin-A (ConA), a T cell mitogen, or lipopolysaccharide (LPS), a B cell mitogen. These data clearly demonstrate the ability of fluorescein-conjugated oligonucleotides to bind to unique cell populations in suspension, allowing simultaneous two-color phenotypic analysis, suggesting that fluorescein-conjugated oligonucleotides may be a useful bridge between *in vitro* molecular biology techniques and *in vivo* cell biology. In addition, these data provide optimism concerning the *in vivo* treatment of chronically infected HIV patients using antisense oligonucleotides.

INTRODUCTION

THE *REV* PROTEIN PRODUCT of the human immunodeficiency virus (HIV) is required for the production of HIV type 1 structural proteins and the production of infectious progeny virus (Holland et al., 1991). A 27-mer phosphorothioate oligonucleotide has been employed as an antiretroviral agent to attenuate viral expression of the human immunodeficiency virus type 1 *in vitro* in chronically infected cells (Matsukara et al.,

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1989). These investigators employed a sequence complementary to the mRNA encoded by the *rev* gene family (*art/trs*). We employed this 27-mer in these studies to provide information on the specificity of cell binding for various lymphoid populations.

The blockage of specific mRNA translation by synthetic oligonucleotides has been demonstrated in cell culture (for reviews see Stein and Cohen, 1988; Zon, 1988; van der Krol et al., 1988). Inhibition of c-*myc* expression has been reported in T lymphocytes and myeloid cells for unmodified antisense DNA oligonucleotides (Heikkila et al., 1987; Holt et al., 1988). In addition, antisense oligonucleotides have been employed to inhibit c-*myb* expression, resulting in attenuated proliferation of normal (Ramsay et al., 1987) and malignant (Barletta et al., 1987) human hematopoietic cells. Recent studies demonstrated that human leukemic hematopoietic cells are more sensitive than normal hematopoietic cells to the inhibition of c-*myb* gene function with antisense oligonucleotides (Calabretta et al., 1991). Finally, uptake of oligonucleotides by lymphoid cells has been demonstrated to be heterogeneous and inducible in cultured murine spleen and lymphoid cells (Krieg et al., 1991). Hence, lymphoid cells may be rational targets for antisense oligonucleotide-based therapy.

Antisense oligonucleotides have been shown to efficiently reduce the replication of human immunodeficiency virus (Matsukara et al., 1987, 1989; Agrawal et al., 1989a, b; Sarin et al., 1988), vesicular stomatitis virus (Lemaitre et al., 1987), Rous sarcoma virus (Stephenson and Zamecnik, 1978), and herpes simplex virus (Smith et al., 1986; Draper et al., 1990). Synthetic oligonucleotides and analogs complementary to viral gene transcripts reduce the infection of cells by different viruses, without significantly affecting normal cell growth (Zamecnik et al., 1986; Lemaitre et al., 1987; Matsukara et al., 1989; Agrawal et al., 1989b). These observations suggest a therapeutically effective oligonucleotide can be demonstrated *in vitro*.

An integral question in the pathway leading to the clinical utility of an oligonucleotide is the availability of the oligonucleotide to the target cells; that is, the oligonucleotide must be available to come in contact with virus within an infected cell. The cellular receptor for HIV is the CD4 antigen found on the surface of T-helper lymphocytes and to a lesser extent on cells of the monocyte-macrophage lineage. The distribution of this surface antigen reflects the tropism of the virus (Dalgleish et al., 1984; Klatzmann et al., 1984; Maddon et al., 1986). Therefore, we used fluorescein-conjugated antisense oligonucleotides with sequence complementary to the *rev* gene of HIV to examine their ability to bind lymphocyte populations of the mouse by two-dimensional flow cytometry.

MATERIALS AND METHODS

Cells

Young adult female BDF_1 mice were obtained from Jackson Laboratories (Bar Harbor, ME) and housed under laminar airflow conditions in the University of Nebraska Medical Center, American Association for Accreditation of Laboratory Animal Care (AALAC)-approved animal quarters. The mice were allowed at least 1 week after shipping to accommodate to the new conditions and had free access to food and water.

Oligonucleotides

The phosphorothioate oligonucleotide is complementary to HIV-1, *rev* a 27-mer (5′TCGTCGGTCTCTC-CGCTTCTTCTTGCC-3′). Approximate absorbances of 10^{-3} M solutions of oligomers were calculated by summing the products of purines × 14 and purines × 7.

The fluorescein-labeled phosphorothioate, FAM-anti-*rev*, was prepared by first synthesizing (6 × 1 μmol) the 5′-aminohexylphosphodiester derivative of this 27-mer sequence (see later) using conventional phosphoramidite chemistry, stepwise sulfurization, and Aminolink 2 (ABI Bulletin 380, Issue No. 49, 1988). To a solution of the crude amino compound (approximately 1000 optical density, OD) in $NaCO_3/NaHCO_3$ (0.5 M,

3.6 ml) was added, with vortexing, a dimethylsulfoxide (DMSO) solution (1.8 ml) of the *N*-hydroxysuccinimidyl esters of the isomeric 5- and 6-carboxyfluorosceins (FAM-NHS, 200 mg; Molecular Probes, Inc., Eugene, OR). After standing overnight in the dark at room temperature, the reaction mixture was divided into six equal volume parts, each of which was then vortexed while adding 5 volumes of absolute EtOH. The resultant combined precipitate of crude FAM-anti-*rev* was applied to a polystyrene high-performance liquid chromatography (HPLC) column (about 25 × 250 mm; Hamilton) and eluted at a flow rate of 13.5 ml/min using 95:5 of 0.1 M aqueous triethylammonium acetate (TEAA) and CH_3CN for 10 min, followed by a linear gradient of increasing CH_3CN (1%/min) for an additional 45 min. The "center cut" of the main peak detected at 260 nm yielded 460 OD of purified FAM-anti-*rev* following precipitation from aqueous NaCl (1 M, 1 ml) and absolute EtOH (5 ml). Analytic HPLC (RP300, ABI; 0–25% CH_3CN in 20 min versus 0.1 M TEAA) with ultraviolet (UV; 260 nm) and fluorescence (480 nm excitation, 520 nm emission) dual monitoring indicated around 80:20 FAM-anti-*rev* and unlabeled oligomer and less than around 1% of other fluorescent materials. The oligomer composition was verified by capillary electrophoresis (ABI Model 270-HT microgel column).

Mitogen stimulation

Single-cell suspension were made from spleen, mesenteric lymph nodes, thymus, and femoral bone marrow. Peripheral blood mononuclear cells were obtained from heparinized blood after axillary exsanguination and gradient separation using Lympholyte-M (CedarLane). Erythrocytes were eliminated by shock lysis using Tris-buffered ammonium chloride, and nucleated cell concentrations were adjusted to 2×10^6 cells per mil in RPMI-1640 containing 10% fetal calf serum, 5×10^{-5} M 2-mercaptoethanol, 2 mM l-glutamine, and penicillin and streptomycin. Mitogens were then added to achieve a final concentration of 10 μg/ml of concanavalin A (ConA) or 100 μg/ml of lipopolysaccharide (LPS). Cells were cultured in the presence of mitogen for 72 h before investigation of their ability to bind fluorescent oligonucleotide.

Flow cytometry

Single-cell suspension (as earlier) were adjusted to 2×10^7 cells per ml in phosphate-buffered saline containing 2% fetal calf serum (PBS-2). Aliquots of 10^6 cells were then incubated with 100 μl FITC-conjugated oligonucleotide (5 nM) for 30 min at 4°C. Excess oligonucleotide was removed by washing twice with PBS-2. Cells for use in double-labeling studies were also incubated with phycoerythrin-conjugated monoclonal antibodies specific for T cells (Thy-1.2, clone 30-H12), CD4 (L3T4, clone GK1.5), and B cells (B220, clone RA3-6B2) for 30 min at 4°C. Excess antibody was then removed by washing as before, and the cells were resuspended and fixed in PBS containing 4% formalin.

Flow cytometric analysis was performed using a FACStar$^+$ (Becton Dickinson) equipped with a 100 mW air-cooled argon laser and the LysysII acquisition and analysis software. List mode data were employed for a minimum of 10^4 collected cells and independent analysis for each sample.

Analysis of oligomer integrity

Cells exposed to the oligonucleotide, in a parallel manner to those empolyed in cell sorting, were centrifuged into a pellet and rinsed with cold saline. The cells were then suspended in a solution of DNA lysis buffer and proteinase K (Applied Biosystems, Inc., Foster City, CA) at 55°C for 2 h. This solution was then mixed with buffer-saturated phenol, chloroform, and water. The genomic DNA was retrieved by ethanol precipitation, and the oligonucleotide retrieved from the supernatant by addition of 10 mM $CdCl_2$ and precipitation. The FAM-labeled oligonucleotide was then examined on an Applied Biosystems Model 373A DNA sequencer. No detection of any breakdown products or loss of oligonucleotide-associated fluorescence was observed (Fig. 1).

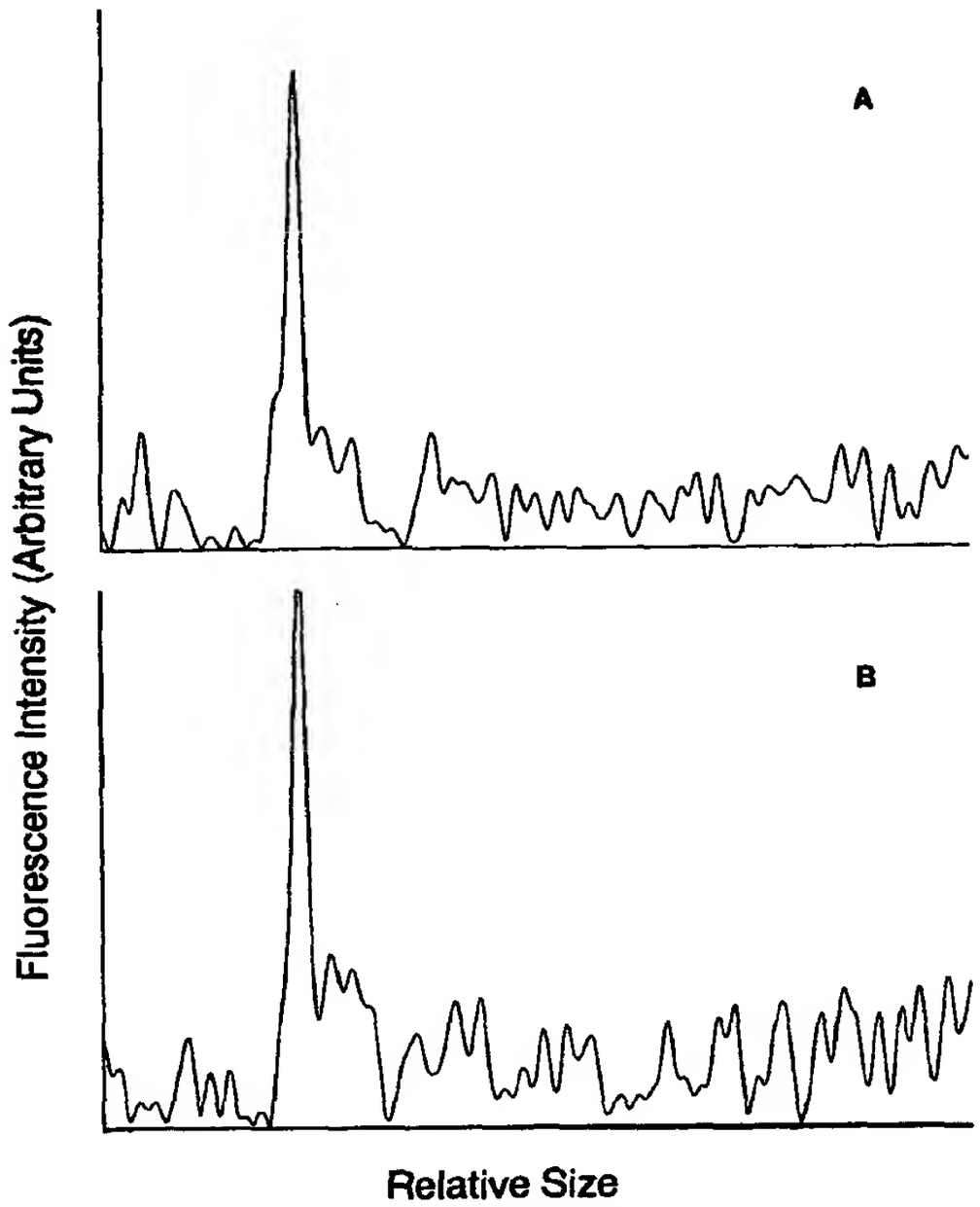

FIG. 1. The fluorescein-conjugated phosphorothioate oligonucleotide retrieved from cells following 2 h of exposure as described in Materials and Methods was analyzed with an Applied Biosystems Model 373A DNA sequence analyzer. (A) Fluorescence intensity on the ordinate versus position in the polyacrylamide gel for a 1 ng sample of oligonucleotide. (B) sample recovered from the washed cell suspension. Little or no degradation can be detected.

RESULTS

To investigate the ability of the oligonucleotide to bind to normal mouse mononuclear cells, single-cell suspensions of spleen, lymph node, bone marrow, thymus, and peripheral blood cells were exposed to FITC-conjugated oligonucleotide (FITC-oligo) for 30 min and subsequently examined by flow cytometry. Significant numbers of cells from lymph node (7.2%), spleen (5.2%), and bone marrow (6.5%) bound the FITC-conjugated oligo (Table 1). In addition, a small but reproducible number of cells (1.7%) in the thymus bound the oligo. No detectable binding was observed with cells from peripheral blood. Two-color analysis using FITC-oligo- and phycoerythrin-labeled monoclonal antibodies to lymphocyte surface antigens was used to determine whether the oligo bound preferentially to specific lymphocyte subsets. Representative contour plots of cells from the lymph node analysis are presented in Fig. 2. The majority of the oligo binding was associated with cells that expressed the 6B2 surface antigen of B lymphocytes (Table 1 and Fig. 2). In the oligo binding population from these tissues, the ratio of $6B2^+$ cells to Thy-1^+ cells was 2:1 in lymph node, 3.5:1 in spleen, and 5.3:1 in bone marrow. Although B cells outnumber T cells in both the spleen and bone marrow, T cells outnumber B cells in the lymph node, and thus the proportional binding by these populations adjusted for the relative proportion of each population in the tissue does not rectify this disproportionate binding pattern.

TABLE 1. FLUORESCEIN-CONJUGATED PHOSPHOROTHIOATE OLIGONUCLEOTIDE BINDING TO CELLS

	% Positive						
Tissue	*Oligo*$^+$	*6B2*$^+$	*Thy-1*$^+$	*L3T4*$^+$	*Oligo*$^+$ *6B2*$^+$	*Oligo*$^+$ *Thy-1*$^+$	*Oligo*$^+$ *L3T4*$^+$
Thymus	1.7 ± 0.4	2.9 ± 0.4	94.3 ± 1.4	89.7 ± 1.3	1.1 ± 3.8	1.3 ± 0.3	1.1 ± 0.4
Spleen	5.2 ± 0.4	63.0 ± 1.1	32.5 ± 1.2	20.4 ± 1.3	3.8 ± 0.3	1.1 ± 0.3	2.4 ± 0.1
Lymph node	7.2 ± 0.5	34.3 ± 21.7	62.6 ± 1.5	41.5 ± 0.9	5.4 ± 0.4	2.7 ± 0.3	2.7 ± 0.3
Bone marrow	6.5 ± 0.4	24.6 ± 0.8	4.9 ± 0.5	5.6 ± 0.5	3.7 ± 0.1	0.7 ± 0.1	1.6 ± 0.2

Since the tissues demonstrating high amounts of oligo binding are associated with B cell proliferation and/or differentiation and the bulk of the binding observed was associated with the B cell population, we speculated that lymphocyte activation or proliferation might reveal a correlation with oligo binding. To test this hypothesis we stimulated cell suspensions from these tissues with the lymphocyte mitogens ConA or LPS. The results of these experiments are presented in Table 2 and Figs. 3 and 4 Mitogen stimulation resulted in a significant increase in the frequency of cells binding the FITC-oligo in these cell populations. The magnitude of the increased frequency of cells binding the FITC-oligo ranged from a 1.4-fold increase in bone marrow stimulated with ConA to a 15.3-fold increase in thymus stimulated with ConA. In every case mitogen-stimulated cells demonstrated a higher frequency of oligo uptake than unstimulated cells. It is important to note, however, that this increased oligo binding was observed not only as an increase in the frequency of cells binding oligo but also as an increase in the amount of oligo bound by individual cells (compare oligo binding in Fig. 4b to that in Fig. 2b). This increase in oligonucleotide binding by the cells was represented by a shift in peak channel fluorescence for the oligonucleotide-positive cells from channel 12.86 for unstimulated cells to channel 39.24 for stimulated cells (Fig. 3). These results demonstrate that there is a significant increase in oligonucleotide binding after mitogenic stimulation, both on the basis of the numbers of cells that bind the oligonucleotide and also on the amount of oligonucleotide bound by these cells.

In addition, it was observed that lymph node cells stimulated with LPS demonstrated significant changes in the expression of the 6B2 surface antigen. As seen in Fig. 5a, after stimulation there were two populations of 6B2$^+$ cells: cells expressing normal amounts of 6B2 (6B2^{hi}) and cells expressing 10-fold less antigen (6B2^{lo}). Figure 5b demonstrates that all the 6B2^{lo} cells bound the FITC-oligo, but only a portion of the 6B2^{hi} cells bound the oligo. In addition, it can be seen in Fig. 5b that the 6B2^{lo} cells generally bound more of the oligo than the 6B2^{hi} cells. It was noted that after stimulation the cells that bound the oligo appeared to be generally small cells on the basis of forward-angle light scatter; however, propidium iodide-stained cells in the S, G_2, and M portions of the cell cycle did not appear to bind oligo preferentially (data not shown). Whether these 6B2^{lo} cells represent a postmitotic population of recently produced B cells is not yet clear.

DISCUSSION

In these studies we incubated freshly isolated murine lymphoid cells at 4°C with 5′ fluorescently labeled phosphorothioate oligonucleotide with sequence complementary to the *rev* transcript of HIV. The purpose was to examine binding of the oligonucleotide to a collection of lymphoid cell lineages to determine differences in oligonucleotide binding to cell lineage and evaluate the dynamics of binding in normal cells and those that were mitogenically stimulated.

The primary conclusions derived from these studies are (1) the binding of oligonucleotide to cells from unstimulated lymphoid tissues was in the order lymph node > bone marrow > spleen > thymus. (2) Cell lineage heterogeneity of binding to unstimulated cells favors cells that express the 6B2 surface antigen. (3)

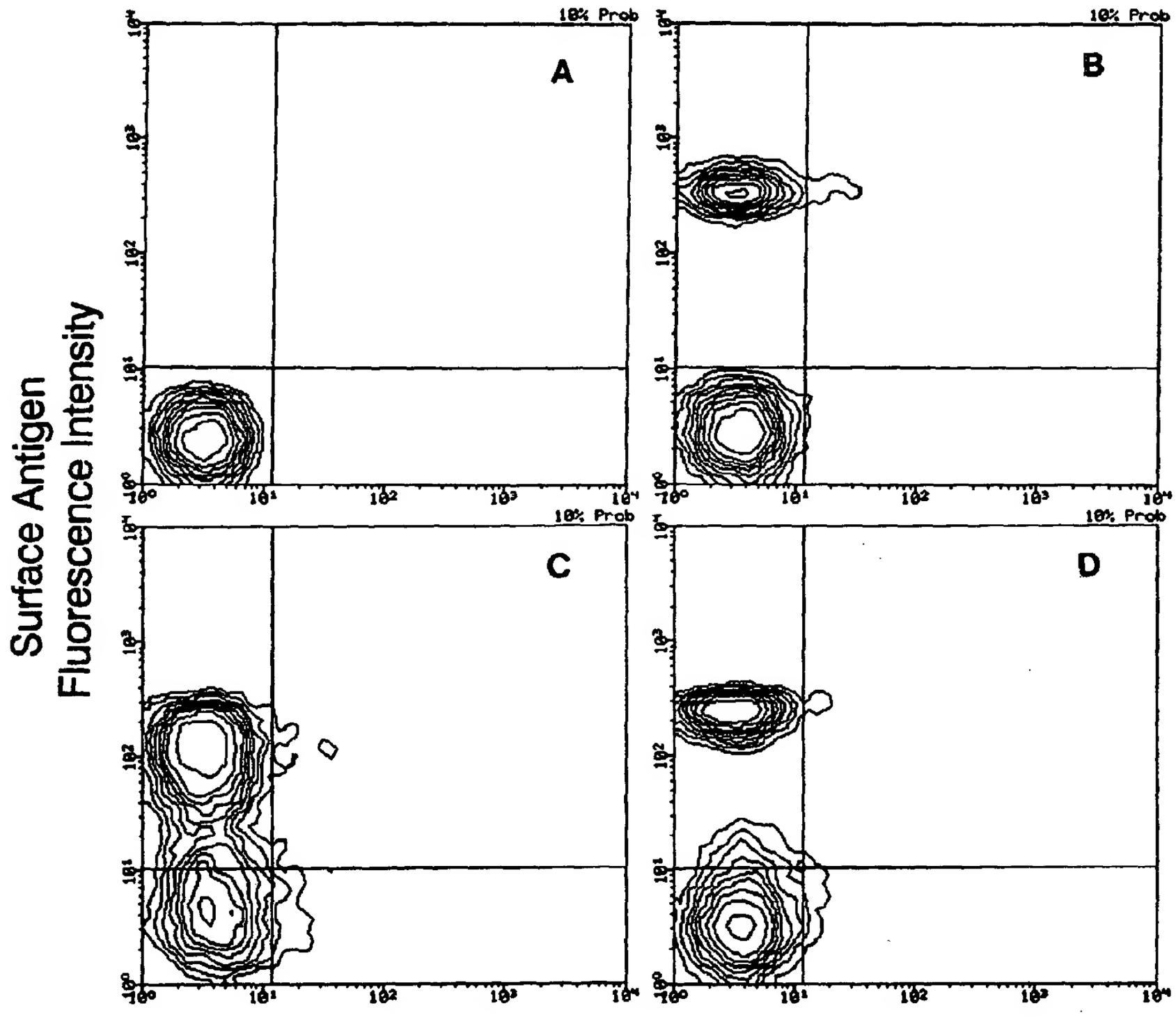

FIG. 2. Two-dimensional flow cytometric contour plot from normal lymph node cells. (A) Background intensity for both phycocrythrin (ordinate) and fluorescein (abscissa). Various antibody markers of surface antigens: 6B2 (B), Thy-1 (C), and L3T4 (D). Cells demonstrating both surface antigen and oligonucleotide binding are plotted in the upper right quadrant of the panels; note that the scale for both axes is logarithmic.

Cells respond to mitogenic stimulation by increasing the number of cells that bind oligonucleotide. The most dramatic evidence was in the lymph node and thymus and for cells that express the Thy-1 surface antigen. (4) The mitogen-stimulated cells represent a novel subpopulation; they are smaller, not as intensely stained by the antigen markers, and not representative of any specific portion of the cell cycle.

The lack of cell lineage homogeneity of oligonucleotide binding is of importance in the consideration of targeting a potentially therapeutic oligonucleotide to the appropriate cell. The data indicate rapidly proliferating cells should make better targets for oligonucleotide binding *in vivo*, and this is especially true for cells of the T cell lineage. Our observation is consistent with the binding of oligonucleotide to primary

TABLE 2. MITOGENS MODULATE PHOSPHOROTHIOATE BINDING TO CELLS

	% Positive						
Treatment	*Oligo*$^+$	*6B2*$^+$	*Thy-1*$^+$	*L3T4*$^+$	*Oligo*$^+$ *6B2*	*Oligo*$^+$ *Thy-1*$^+$	*Oligo*$^+$ *L3T4*$^+$
LPS							
Thymus	10.6	7.0	99.2	95.1	3.8	13.2	7.6
Spleen	9.8	72.7	41.8	30.2	9.1	2.9	2.4
Lymph node	28.7	39.4	88.3	64.1	23.6	22.6	16.0
Bone marrow	11.3	45.8	16.5	13.8	8.4	3.9	4.0
ConA							
Thymus	26.0	19.2	99.4	89.8	10.5	29.6	20.9
Spleen	10.2	87.8	65.6	42.0	9.9	3.7	2.2
Lymph node	27.8	55.6	79.2	44.4	43.4	19.3	8.1
Bone marrow	8.8	32.1	12.9	10.1	7.2	3.9	3.1

lymphoid cells cultured for 48 h (Krieg et al., 1991). This observation may explain why c-*myb* antisense oligonucleotides are more effective in leukemic than normal cells (Calabretta *et al.*, 1991). Finally, these data provide optimism about the treatment of HIV infections with the antisense *rev* phosphorothioate oligonucleotide, since more rapidly dividing cells are better hosts for HIV replication.

Oligonucleotide binding to the cell surface is an obligatory initial step in the cellular availability of effective antisense oligonucleotide therapy. Mammalian cells may internalize oligonucleotides and their derivatives by an endocytotic mechanism, and undegraded oligonucleotides can be retrieved from cellular nuclei and

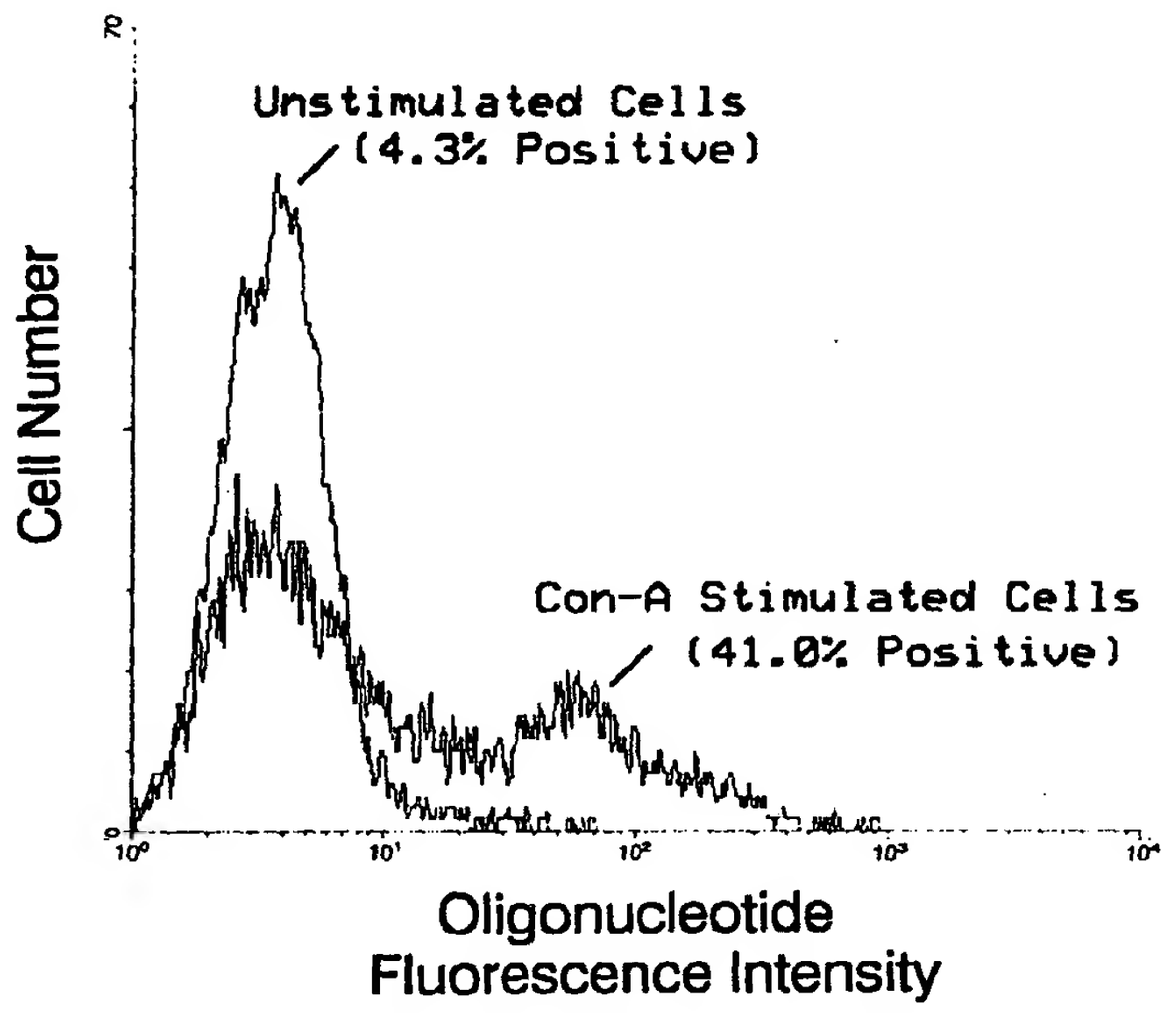

FIG. 3. A frequency histogram of cell number on the ordinate plotted versus fluorescent intensity on the abscissa. A single peak is evident in the normal lymph node cells, and a bimodal distribution is seen in the mitogen-stimulated lymph node cells.

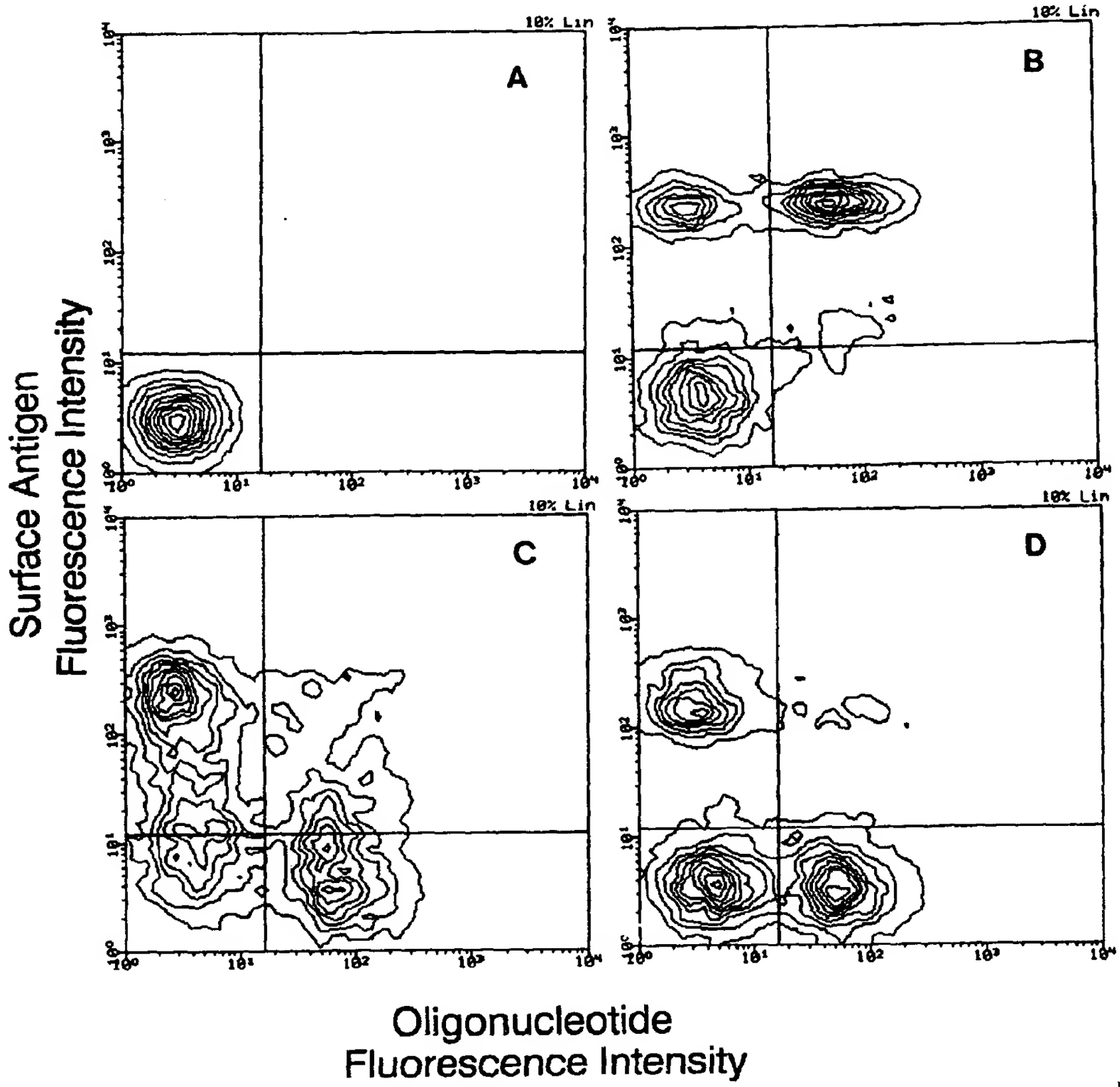

FIG. 4. Two-dimensional flow cytometry from ConA mitogen-stimulated lymph node cells. (A) Background intensity for both phycoerythrin (ordinate) and fluorescein (abscissa). Various antibody markers of surface antigens: 6B2 (B), Thy-1 (C), and L3T4 (D). Cells demonstrating both surface antigen and oligonucleotide binding are plotted in the upper right quadrant of the panels; note that the scale for both axes is logarithmic.

cytoplasm (Yakubov et al., 1989; Loke et al., 1989; Stein et al., 1988; Leonetti et al., 1990). The mechanism of oligonucleotide uptake may involve a specific receptor (Neckers, 1989) located in the plasma membrane. The transport and cellular uptake of synthetic oligonucleotides has been examined by several investigators. Ho et al. (1990) demonstrated the efficacy of phosphorothioate oligonucleotides in preventing the expression of transferrin receptor in HL60 cells and that the efficacy is a function of serum stability and cellular uptake (Ho et al., 1989). The uptake of the oligonucleotide can be enhanced through conjugation with poly-l-lysine (Leonetti et al., 1990) or antibody-targeted liposomes (Leonetti et al., 1990). However, measures to

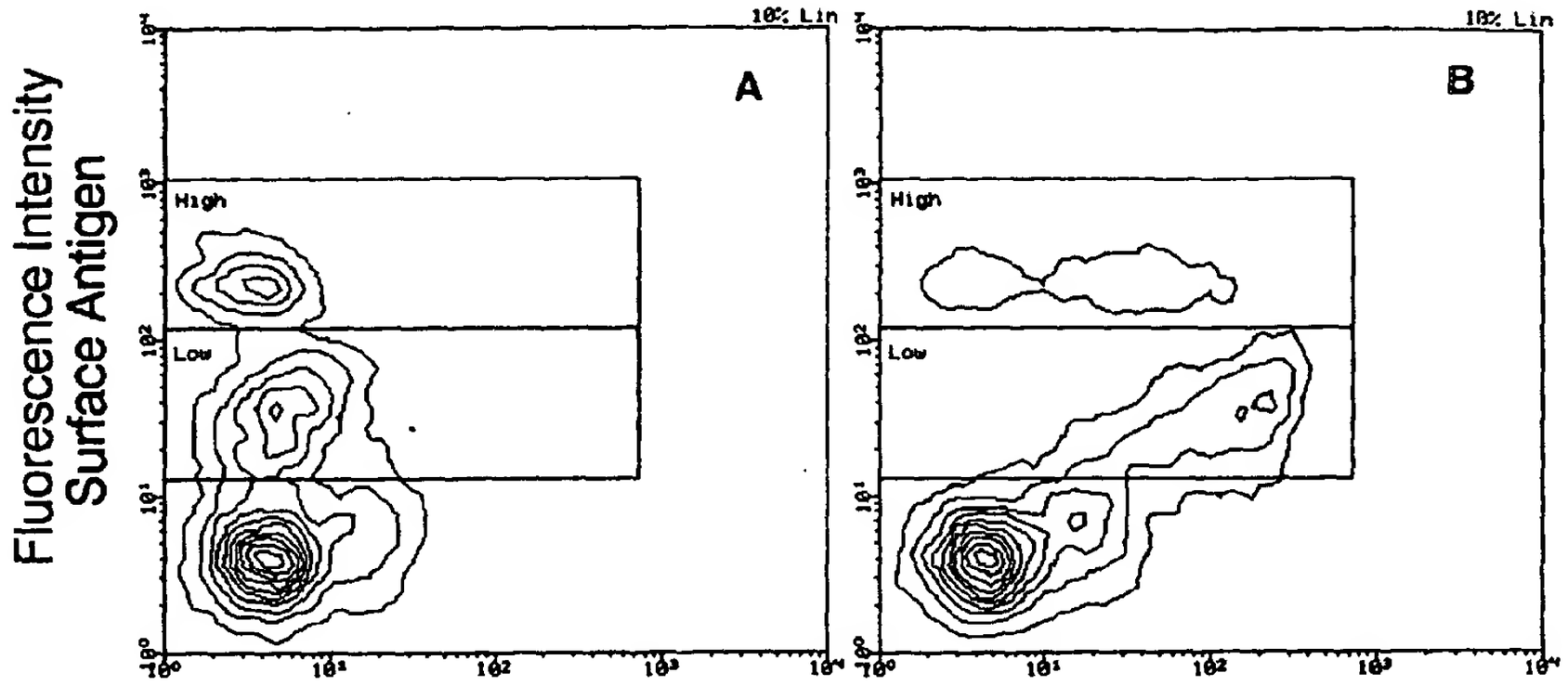

FIG. 5. *Two-color flow cytometric contour plot of LPS-stimulated cells with 6B2 surface marker (ordinate) versus oligonucleotide fluorescence intensity (abscissa): (A) 6B2 surface antigen with no oligonucleotide; (B) 6B2 surface marker with oligonucleotide.*

enhance uptake may not be necessary because an apparent transport system exists to move oligonucleotides into cells (Yakubov et al., 1989).

The subcellular transport of oligonucleotides was investigated by Chin et al. (1990) and Leonetti et al. (1991) in studies in which the oligonucleotide was microinjected into cell cytoplasm. They observed a very rapid (2–10 min) transport of oligonucleotide from the site of injection to the cell nucleus.

Studies have been conducted to examine the single-injection pharmacokinetics of phosphorothioate oligonucleotides in the mouse (Agrawal et al., 1991) and rat (Iversen et al.), which indicate sufficient bioavailability that further therapy should be considered. Further work conducted in this laboratory indicates that repeated injections into the mouse substantiate the prolonged (half-life of approximately 36 h) residence time of a phosphorothioate *in vivo*. However, these studies allow evaluation of parameters related to the whole body but offer little insight into the bioavailability of the oligonucleotide to the target cell or cells. Specifically, the antisense *rev* oligonucleotide must be available to the potentially infected lymphoid cells for future therapy to be considered. The data reported here offer significant additional information that lymphoid cells can bind the oligonucleotide *in vitro*; hence oligonucleotide therapy for HIV infection should receive continued interest.

Studies are currently underway to examine the antisense *rev* oligonucleotide interactions with murine lymphoid cells *in vivo*. These studies will be combined with single-injection pharmacokinetic studies, repeated-injection pharmacokinetics, and constant-infusion studies *in vivo*, which provide quantitative and qualitative information regarding the bioavailability of phosphorothioate oligonucleotides. Thus, the dose required to provide a specific concentration of intact oligonucleotide to target tissue can be cominbed with the rate and extent of oligonucleotide binding and eventual entry into lymphoid cell lineages. Finally, these data will provide the basis for predicting the *in vivo* efficacy of antisense therapy.

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